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RESEARCH ARTICLE

The role of m6A-modified CircEPHB4 in glioma pathogenesis: Insights into cancer stemness metastasis

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Abstract

Objective: While existing literature has provided insights into involvement of circEPHB4, SOX2 in glioma, their precise molecular mechanisms and synergistic implications in glioma pathogenesis still dim. This study aims to investigate significance and underlying mechanism of m6A-modified circEPHB4 in regulating SOX2/PHLDB2 axis in gliomas. **Methods**: The mRNA and protein expression were tested by qRT-PCR and Western blot, respectively. ChIP assay was performed to detect SOX2 enrichment on the PHLDB2 promoter. Cell sphere-forming assay to detect self-renewal ability, flow cytometry to determine positivity of CD133 expressions, Malignant behavior of glioma cells were detected by CCK-8, plate colony formation, scratch, and transwell assays. Glioma xenograft models were constructed to investigate effects of CircEPHB4 in tumor development in vivo. **Results**: Methyltransferase MELLT3 upregulated m6A modification of CircEPHB4, and YTHDC1 promoted cytoplasmic localization of m6A-modified CircEPHB4. Overexpression of wild-type CircEPHB4 enhanced glioma cells' stemness, metastasis, and proliferation. Cytoplasmic CircEPHB4 increased SOX2 mRNA stability by binding to IGF2BP2, and the effects observed by SOX2 knockdown were reversed by CircEPHB4 in glioma cells. SOX2 promoted transcriptional expression of PHLDB2 by enriching the PHLDB2 promoter region. SOX2 reversed the inhibition of PHLDB2 knockdown on stemness of glioma, cell proliferation, and metastasis. In vivo experiments also revealed that CircEPHB4 upregulated PHLDB2 expression by stabilizing SOX2 mRNA, which promoted in vivo tumor growth and accelerated stemness of glioma cells and metastasis. **Conclusion**: This study reveals functional interaction and molecular mechanisms of m6A-modified circEPHB4 in regulating SOX2/PHLDB2 axis, highlighting their importance in glioma pathogenesis and potential as therapeutic targets.

Introduction

Glioma is a highly prevalent and devastating intracranial malignancy that accounts for 30% of all brain tumors.[1] Despite current standard treatments involving adjuvant radiochemotherapy following surgical resection, the prognosis of the patients remains dismal,[2] with patients with high-grade gliomas exhibiting a median overall survival time of 15–36 months.[3] Glioma relapse and progression are often attributed to residual infiltrative tumor cells that evade surgical removal or postoperative therapy, leading to radiotherapy/chemotherapy resistance and continued proliferation.[4] Notably, cancer stem cells, characterized by their persistent self-renewal, proliferation and diverse differentiation potential into different biological phenotypes, contribute to glioma growth, metastasis, and treatment resistance, warranting the identification of effective therapeutic targets.[5–7]

Emerging evidence suggests the involvement of circular RNAs (circRNAs) in the regulation of glioma cell proliferation, invasion and stemness,[8–10] with abnormal circRNA expression associated with the aggressive nature of glioblastoma.[11] As a result, elucidating the potential molecular mechanisms via which glioma cells interact

with circRNAs could help determine more effective treatment targets for treating gliomas.

Ephrin type-B receptor (EphB) refers to the EphB family of receptor tyrosine kinases, which are a subclass of the Eph receptor family.[12] Eph receptors and their ligands, ephrins, play important roles in various cellular processes such as cell migration, adhesion, and tissue development, and EphB receptors have been implicated in neural development, cell aggregation, migration, and angiogenesis.[13] EphB4 is a specific member of the EphB receptor family and has been implicated in various cancers, including glioma, and has been shown to play a critical role in tumor progression by controlling cell proliferation, differentiation, and migration.[14,15] Aberrant regulation of EphB4 has been associated with enhanced tumor progression by controlling cell proliferation, differentiation, and migration.[16] Circular RNA EPHB4 (circEPHB4) is a circRNA derived from the *EPHB4* gene and reported to be a regulator of cancer progression.[17] Previous studies revealed that CircEPHB4 could regulate the proliferation, invasion, and stemness of glioma cells.[5,18] Additionally, our preliminary bioinformatic analysis (unpublished data) using the CircInteractome database revealed a potential binding between CircEPHB4 and IGF2BP2 protein, and further analysis of the Starbase database highlighted the binding of IGF2BP2 to SRY-box transcription factor 2 (SOX2) messenger RNA (mRNA) and showed that it was important in maintaining the essential stem properties of glioma stem cells.[19] The functional association of SOX2 with glioma cell stemness has been well-established and correlated with unfavorable prognosis.[20–22] Although there has been significant research on CircEPHB4 and SOX2 separately, their relationship in the malignant behavior of glioma cells has not been addressed.

Moreover, transcription factors are known to be crucial in regulating downstream target genes' transcriptions by linking them to their promoters. Among them, the PH domain-containing protein Pleckstrin Homology Like Domain Family B Member 2 (PHLDB2) is a significant mediator of cell migration[23] and has been reported to be functionally related to epithelial-mesenchymal transformation (EMT), a critical process for cancer metastasis and stem cells.[24] Our preliminary bioinformatic analysis revealed that the PHLDB2 promoter region contains a binding site that can be targeted by SOX2. However, since the targeting relationship between SOX2 and PHLDB2 in this context has not been previously studied, we postulate that the circEPHB4/SOX2/PHLDB2 signaling axis could potentially regulate the behavior of glioma cells, which may provide a new avenue for further investigation into the role of these molecules in glioma pathogenesis and potential therapeutic strategies.

Herein, we designed this present study to comprehensively investigate the roles and underlying mechanism of CircEPHB4 and SOX and the significance of the SOX2/PHLDB2 axis to understand the role of CircEPHB4, its m6A modification and its interaction with SOX2 and PHLDB2 in the malignant behavior of glioma to provide insights into potential therapeutic targets and strategies for enhancing glioma treatment.

Material and Methods

This section is detailed in the supplementary material.

Results

Characterization of CircRNA EPHB4 in glioma cells

A previous study reported increased CircRNA EPHB4 (hsa_circ_0081519) expression in glioma tissues relative to paracancerous tissues (n = 40) and the association of EPHB4 with poor patients' survival.[5] Herein, we assessed CircRNA EPHB4 expressions in various glioma cell lines and similarly detected overexpression of CircRNA EPHB4 in all five cancerous cells (greatest expression, SHG44 cells; lowest expression, A172 cells) compared to non-glioma cells SVGP12 (Fig. 1A). Thus, SHG44 and A172 cells were used for the following experimentation.

Circbase database (http://www.circbase.org/) analysis indicated chr7:100400186-100417918 as the location of CircRNA EPHB4 mature cleavage, which had a total length of 3052 bp, and generated from reverse cleavage of exon 5 and exon 17 of the gene EPHB4 transcript (Fig. 1B). Sanger sequencing results indicated that sequences containing reverse cleavage sites of CircEPHB4 could be obtained by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (divergent primers) (Fig. 1B). Results of agarose gel electrophoresis experiments showed that only convergent primers could reach the amplification product, whether complementary DNA (cDNA) or gDNA was used as a template. However, the CircRNA EPHB4 product could not be obtained using genomic DNA (gDNA) as a template, and the circular sequence of circRNA EPHB4 could be obtained using cDNA as a template (Fig. 1C). Ribonuclease R (RNase R) significantly reduced the mRNA level of EPHB4 in glioma cells but did not affect CircRNA EPHB4 expression (Fig. 1D). After treatment with actinomycin D, we observed a significant reduction in EPHB4 mRNA levels with increased treatment time, while no significant change in CircRNA EPHB4 expression was observed (Fig. 1E), further illustrating the characteristics of CircEPHB4. Nucleocytoplasmic isolation and RNA fluorescence in situ hybridization (FISH) experiments indicated

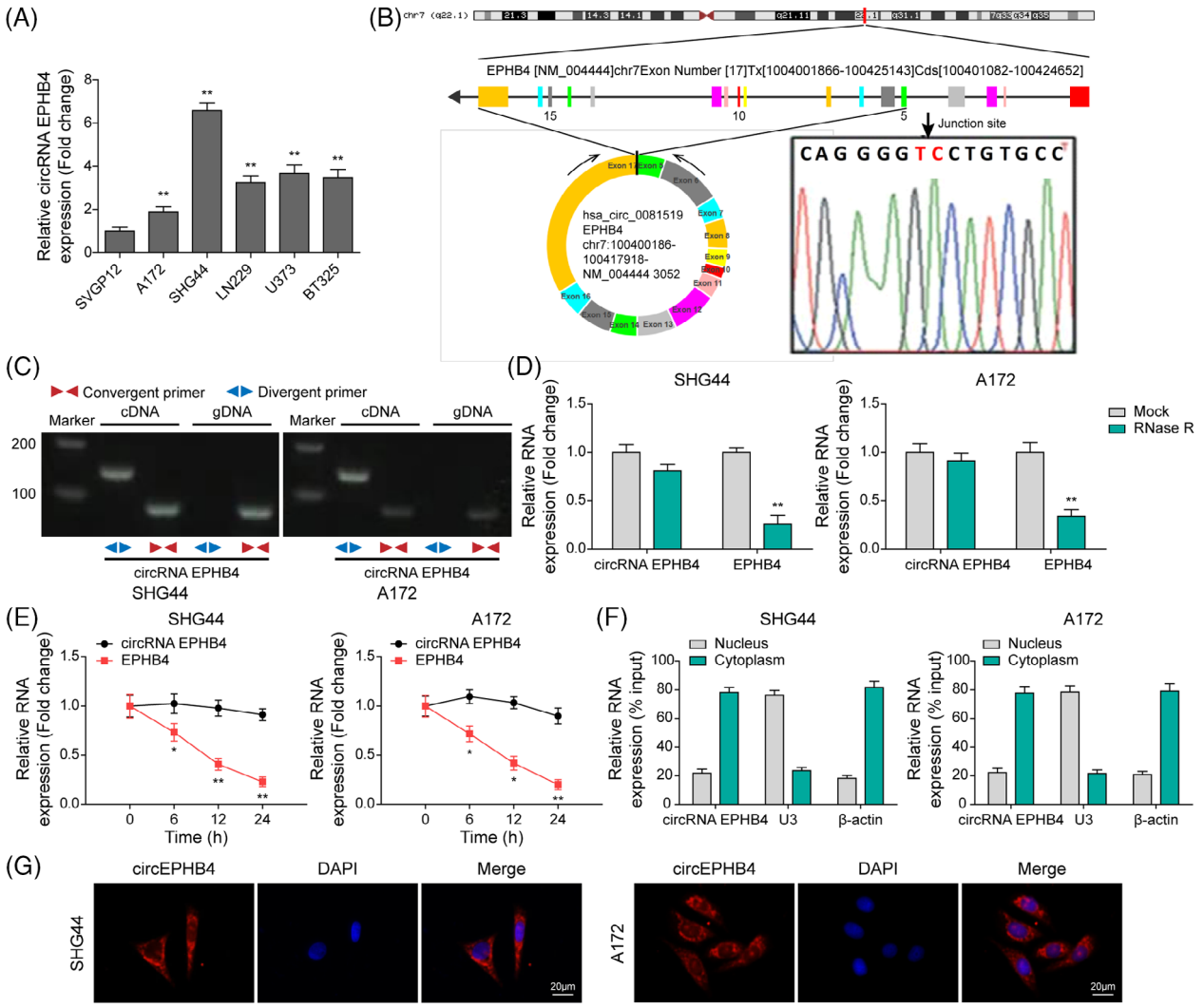

Figure 1. Characterization of CircEPHB4 in glioma cells. (A) CircEPHB4 expression in glioma cells detected by qRT-PCR; * indicates $p < 0.05$ versus SVGP12 cell lines; (B) CircEPHB4 chromosomal localization (top) and Sanger sequencing results (bottom); (C) Agarose gel electrophoresis results of cDNA and gDNA amplified with divergent primers and convergent primers in SHG44 and A172 cells; (D) After extracting total RNA and treated using RNase R, linear EPHB4 mRNA and CircEPHB4 expressions were determined via qRT-PCR; * indicates $p < 0.05$ compared with Mock; (E) SHG44 and A172 cells treatment in actinomycin D, abundance of linear EPHB4 mRNA and CircEPHB4 in SHG44 and A172 via qRT-PCR; *$p < 0.05$, **$p < 0.01$; (F) Localization of CircEPHB4 in SHG44 and A172 cells via nucleoplasmic separation, with β-FISH and U3 as positive controls, respectively; (G) Localization of CircEPHB4 in SHG44 and A172 cells via RNA FISH, with a scale of 20 μm. All experiments were performed thrice.

the presence of CircEPHB4 in the cytoplasm and nucleus of the cells (Fig. 1F,G).

Collectively, these findings indicate that CircEPHB4 is a circRNA that is abundantly and stably expressed in glioma cells.

YTHDC1 promoted cytoplasmic localization of m6A-modified CircEPHB4

To explore the molecular mechanism of CircRNA EPHB4 expression in glioma, the abundance of m6A methylation sites of CircRNA EPHB4 was predicted on the SRAMP website, following which 30 potential sites were identified (Fig. 2A, Annex 1). To determine the effective m6A methylation fragment in circEPHB4, we constructed three pairs of primers for amplifying the top three m6A methylation sites (sites 9, 17, and 19) in the CircEPHB4 sequence (Annex 1). The MeRIP results indicated that the m6A antibody was able to precipitate CircEPHB4, and only site 17 (AUGCU GGACU GUUGG) showed high methylation levels in the five investigated glioma cell lines compared with the non-tumor cell lines (Fig. 2B, Fig. S1).

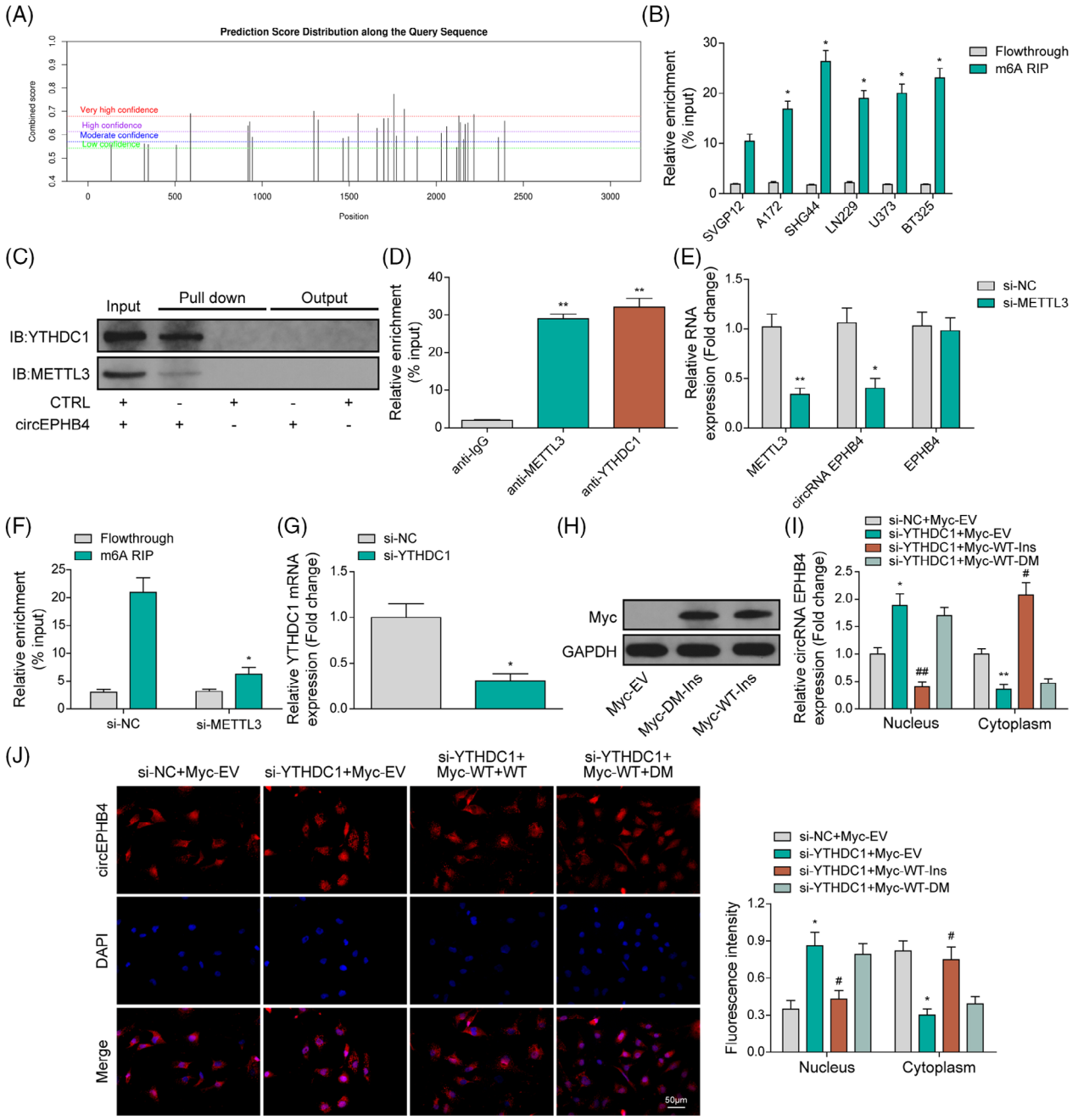

Figure 2. YTHDC1 affected the cellular localization of m6A-modified CircEPHB4. (A) The abundance of m6A methylation site of CircEPHB4 predicted by SRAMP website; (B) m6A methylation level of CircEPHB4 site 17 via MeRIP; * indicates $p < 0.05$ versus SVGP12 cells; (C) CircEPHB4 binding to MELLT3 and YTHDB1 protein via RNA pull down assay; (D) Binding of MELLT3 and YTHDC1 antibodies to CircEPHB4 in SHG44 cells detected by RIP assay; * indicates $p < 0.05$ versus anti-IgG; (E) MELLT3 mRNA, EPHB4 mRNA and CircEPHB4 abundance in SHG44 cells via qRT-PCR; *$p < 0.05$ versus si-NC; (F) m6A methylation level of CircEPHB4 site 17 in SHG44 cells detected by MeRIP; *$p < 0.05$ versus si-NC; (G) YTHDC1 levels in SHG44 cells assessed via qRT-PCR; (H) YTHDC1-WT and YTHDC1-DM overexpression in SHG44 cells assessed via Western blot; (I) CircEPHB4 localization in SHG44 cells assessed via nucleocytoplasmic separation; *$p < 0.05$ versus si-NC + Myc-EV, and $^{\#}p < 0.05$ versus si-YTHDC1 + Myc-EV; (J) The localization of CircEPHB4 in SHG44 cells detected by RNA FISH, with a scale of 50 μm; * indicates $p < 0.05$ compared with si-NC + Myc-EV, and # indicates $p < 0.05$ compared with si-YTHDC1 + Myc-EV. YTHDC1-WT sequence: GAACU; YTHDC1-DM sequence: CUUGA. All experiments were performed thrice.

Thus, CircEPHB4 could be modified by m6A, and site 17 in the sequence had elevated m6A methylation levels in glioma cells.

The methyltransferase MELLT3 mediated the m6A modification of CircRNA.[25] RNA pulldown results showed that CircEPHB4 precipitated MELLT3 protein (Fig. 2C). RNA immunoprecipitation (RIP) experiments demonstrated that the MELLT3 antibody enriched CircEPHB4 (Fig. 2D). Meanwhile, the expression of CircRNA EPHB4 and the level of m6A modification were significantly decreased after the knockdown of MELLT3 (The transfection efficiency results of si-MELLT3 were shown in Fig. S2), while no change was observed in EPHB4 mRNA levels (Fig. 2E,F). These results suggest that the methyltransferase MELLT3 could upregulate the m6A modification of CircEPHB4 and promote its stability.

The YTH domain containing 1 (YTHDC1) protein promoted the cytoplasmic localization of m6A-modified CircRNA.[25] RNA pulldown results showed that CircEPHB4 precipitated the YTHDC1 protein (Fig. 2C), which in turn enriched CircEPHB4 (RIP experiments, Fig. 2D). Nucleocytoplasmic separation experiments demonstrated that YTHDC1 knockdown increased CircEPHB4 content in the nucleus and decreased CircEPHB4 content in the cytoplasm and could be reversed by overexpressing wild-type YTHDC1 (YTHDC1-WT, sequence: GAACU), while the overexpression of m6A binding to defective YTHDC1 mutant (YTHDC1-DM, sequence: CUUGA) did not cause similar effects (Fig. 2G–I). RNA FISH experiment further demonstrated that overexpressing YTHDC1-WT could reverse the YTHDC1-knockdown-led nuclear accumulation of CircEPHB4, while YTHDC1-DM overexpression could not lead to similar effects (Fig. 2J). Collectively, the YTHDC1 protein could promote the cytoplasmic localization of CircEPHB4 by binding to m6A-modified CircEPHB4.

m6A-modified CircEPHB4 promoted stemness of glioma cell

Here, we assessed circEPHB4 impact with m6A modification on glioma cells by constructing a CircEPHB4 (AUGCU GGGCU GUUGG) overexpression plasmid with site 17 mutation. Nucleocytoplasmic separation experiments indicated an increase in CircEPHB4 levels in the cytoplasm and nucleus after wild-type CircEPHB4 overexpression (oe-CircEPHB4), while overexpression of mutant CircEPHB4 (oe-CircEPHB4 Mut) led to the localization of CircEPHB4 in the nucleus (Fig. 3A,B). MeRIP experiments demonstrated a significant decrease in CircEPHB4 m6A modification level following mutant CircEPHB4 overexpression compared to wild-type CircEPHB4 overexpression (Fig. 3C). Cell sphere-forming assays showed an increase in the number of glioma cell spheres with wild-type CircEPHB4 overexpression, while no significant change was observed upon mutant CircEPHB4 overexpression (Fig. 3D). Furthermore, overexpressing wild-type CircEPHB4 led to greater positivity of stemness marker CD133, while no effects were observed following the overexpression of mutant CircEPHB4 on flow cytometry (Fig. 3E,F). Moreover, the levels of stemness markers Nestin, Nanog, Oct4, CD44, and CD133 were increased in glioma cells after overexpressing wild-type CircEPHB4 but not with mutant CircEPHB4 overexpression (Fig. 3G). Taken together, m6A modification increased CircEPHB4 cytoplasmic localization and promoted the self-renewal of glioma cells.

m6A-modified CircEPHB4 promoted glioma cell proliferation, invasion, and migration

MTT assay showed that the overexpression of wild-type CircEPHB4 enhanced the cell viability of glioma cells, while no effects were observed upon overexpressing mutant CircEPHB4 (Fig. 4A). Colony formation assay demonstrated that overexpression of wild-type CircEPHB4 rather than mutant CircEPHB4 promoted glioma cells' colony formation ability (Fig. 4B). The overexpression of wild-type CircEPHB4 promoted glioma cell invasion and migration but not mutant CircEPHB4 overexpression (Fig. 4C–E). On Western blot, we observed a decrease in epithelial-mesenchymal transition (EMT) marker E-Cadherin and an increase in N-Cadherin and vimentin (VIM) levels after overexpressing wild-type CircEPHB4 in glioma cells, but not with mutant CircEPHB4 overexpression (Fig. 4F). Thus, m6A-modified CircEPHB4 was localized in the cytoplasm and promoted glioma cell proliferation, invasion, and metastasis.

Cytoplasmic circEPHB4 increased SOX2 mRNA stability by binding to IGF2BP2

CircInteractome database analyses showed that CircEPHB4 could bind to the IGF2BP2 protein (Annex 2), which was then confirmed via RIP and RNA pulldown assays (Fig. 5A,B). Immunofluorescence and RNA FISH experiments showed that such interaction occurred in the cytoplasm (Fig. 5C). IGF2BP2 is reported to be essential for mRNA stability.[26] The Starbase database analysis also showed an interaction between IGF2BP2 and SOX2 mRNA. Basic Local Alignment Search Tool (BLAST) sequencing analysis showed a direct bonding potential between CircEPHB4 and the mRNA 3′UTR of SOX2 with AU-Rich Elements at the AAACA site. RNA pulldown (Fig. 5D) and RIP assays (Fig. 5E) demonstrated that CircEPHB4 and the IGF2BP2 antibody could precipitate the

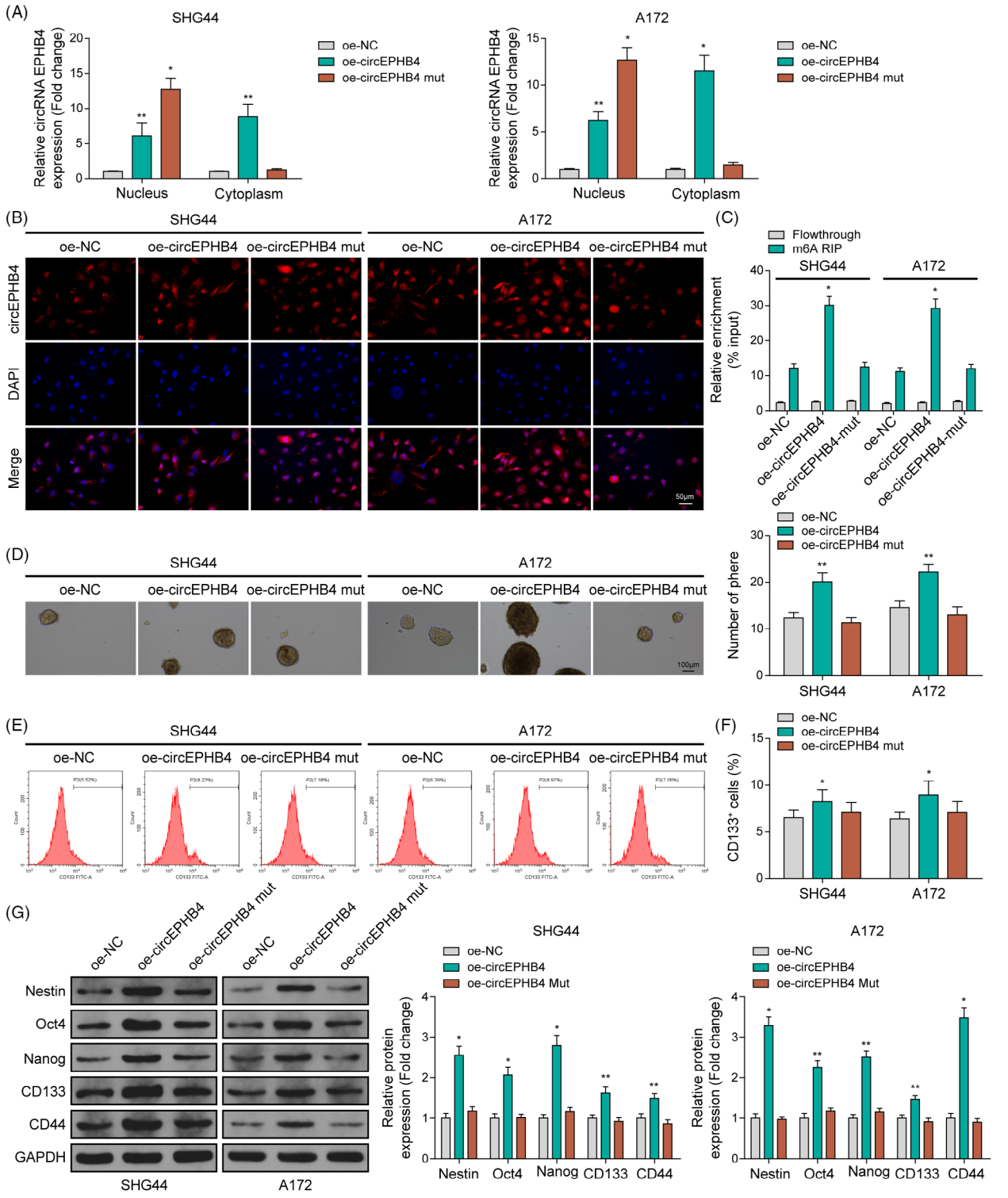

Figure 3. m6A-modified CircEPHB4 affected stem properties in glioma cells. (A and B) The localization of CircEPHB4 assessed via (A) nucleocytoplasmic separation, and (B) RNA FISH, with a scale of 50 μm; (C) The m6A methylation level of CircEPHB4 site 17 via MeRIP; (D) Representative images and quantitative results of glioma cell spheres formed by SHG44 and A172 cells in cell sphere-forming assay, with a scale of 100 μm; (E and F) The positive rate of CD133 on SHG44 and A172 cells via flow cytometry; (G) Related genes' protein levels determined via Western blot. *$p < 0.05$ versus oe-NC. The experiments were performed thrice.

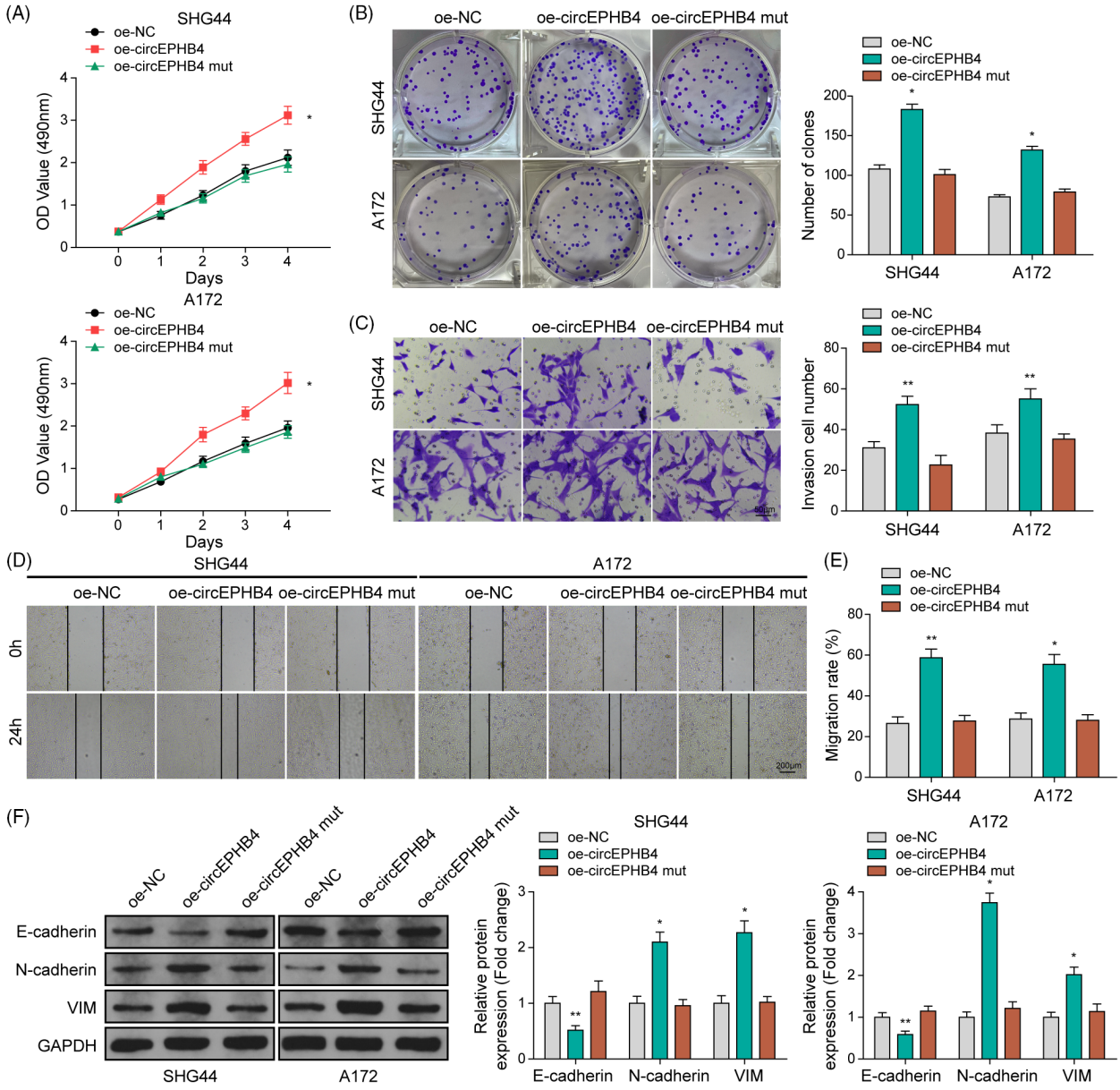

Figure 4. m6A-modified CircEPHB4 affected the proliferative, invasive, and metastatic ability of SHG44 and A172 cells. (A) Cell viability via MTT assay; (B) Cell proliferation via colony formation assay; (C) Cell invasion via transwell assay, with a scale of 50 μm; (D and E) Cell migration via cell scratch assay, with a scale of 200 μm; (F) Related genes' protein levels determined via Western blot. * indicates $p < 0.05$ compared with oe-NC. All cell experiments were repeated three times.

SOX2 mRNA 3′UTR, respectively. Further experiments showed overexpression of SOX2 in both glioma cell lines and tissues (Fig. 5F,G), as well as a positive correlation of SOX2 mRNA expression level with CircEPHB4 expression (Fig. 5H). In addition, to further investigate whether the CircEPHB4/IGF2BP2 complex was essential for SOX2 mRNA stabilization, luciferase reporter plasmids containing wild-type SOX2-3′UTR (SOX2-WT) or mutant 3′UTR (SOX2-Mut) were constructed, and we found that overexpressing CircEPHB4 promoted SOX2-WT luciferase activities and did not affect those of SOX2-Mut (Fig. 5I). Subsequently, we performed qRT-PCR to detect the expression of SOX2 mRNA in glioma cells transfected with oe-circEPHB4. The results manifested that overexpression of circEPHB4 promoted the expression of SOX2 mRNA (Fig. 5J). After treating glioma cells with actinomycin D, the content of ANXA2 mRNA was measured at several intervals, and the results showed decreasing SOX2

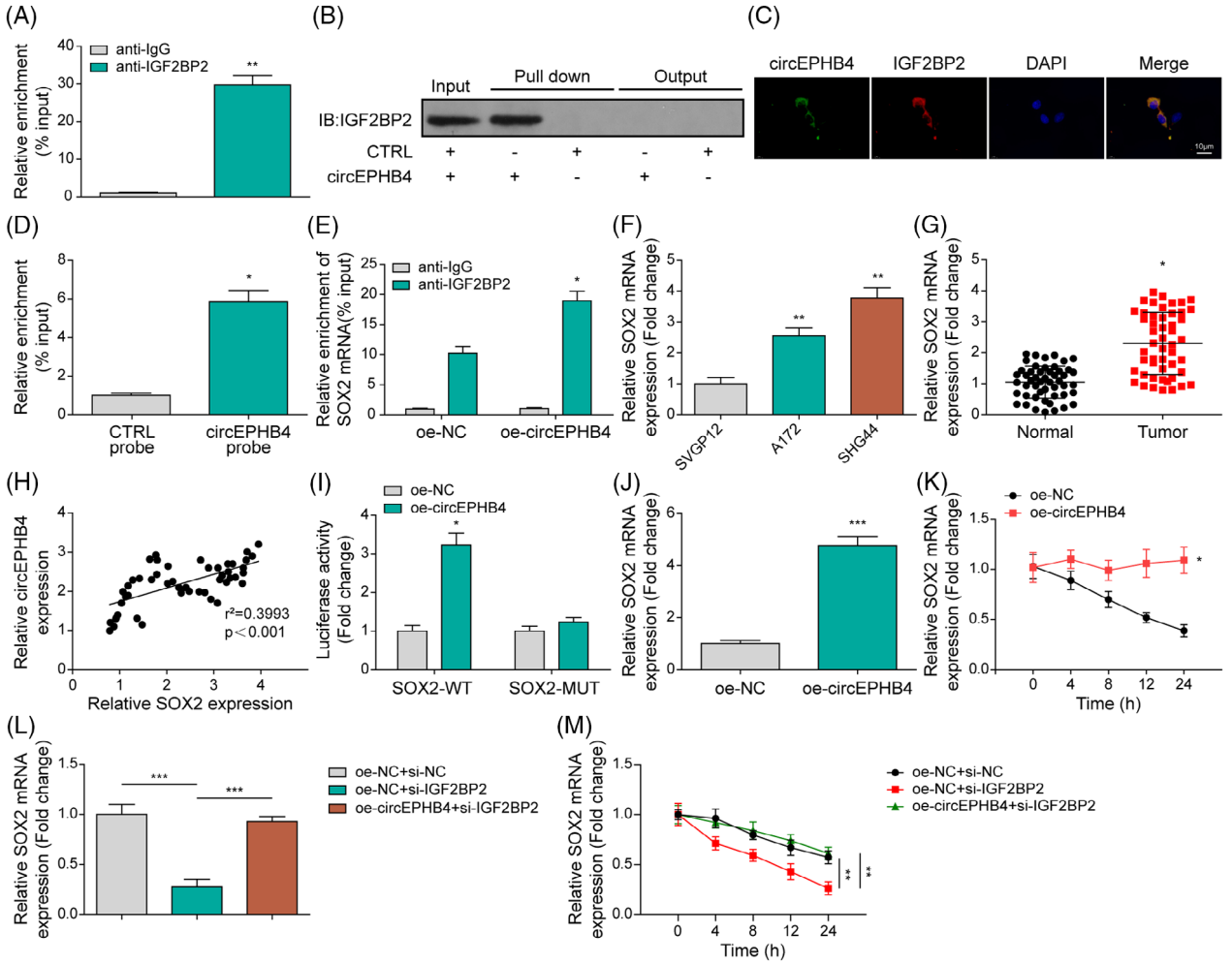

Figure 5. Cytosolic CircEPHB4 binding to IGF2BP2 affected the stability of SOX2 mRNA. (A) The binding of CircEPHB4 to IGF2BP2 in SHG44 cells assessed via RIP; *$p < 0.05$ versus anti-IgG; (B) CircEPHB4 binding to IGF2BP2 in SHG44 cells assessed via RNA pulldown assay; (C) CircEPHB4 and IGF2BP2 protein co-localization in SHG44 cells assessed via immunofluorescence and RNA FISH, with a scale of 10 μm; (D) SOX2 mRNA binding to CircEPHB4 in SHG44 cells assessed via RNA pulldown assay; *$p < 0.05$ versus CTRL; (E) SOX2 mRNA binding to IGF2BP2 in SHG44 cells via RIP; *$p < 0.05$ versus anti-IgG; (F) SOX2 expression via qRT-PCR; *$p < 0.05$ versus SVGP12 cells; (G) SOX2 expression in glioma and adjacent normal tissues assessed via qRT-PCR; *$p < 0.05$ versus Normal; (H) Association of SOX2 mRNA with CircEPHB4 expression in glioma tissues determined by Pearson correlation coefficient analysis; (I) mRNA 3′UTR binding of CircEPHB4 to SOX2 via luciferase reporter assay; *$p < 0.05$ versus oe-NC; (J) SOX2 mRNA expression in SHG44 cells assessed via qRT-PCR; ***$p < 0.001$ versus oe-NC. (K) SHG44 cells treatment in actinomycin D, abundance of SOX2 mRNA was detected by qRT-PCR; *$p < 0.05$ versus oe-NC; (L) SHG44 cells transfected with oe-circEPHB4 and/or si-IGF2BP2 and SOX2 mRNA expression measured by qRT-PCR; ***$p < 0.001$; (M) SHG44 cells treatment in actinomycin D, abundance of SOX2 mRNA was detected by qRT-PCR; **$p < 0.01$. The experiments were performed thrice.

mRNA degradation following CircEPHB4 overexpression (Fig. 5K). Furthermore, the results from Figure 5L demonstrated that knockdown of IGF2BP2 (The transfection efficiency results of si- IGF2BP2 were shown in Fig. S2) inhibited mRNA expression of SOX2 and partly reversed the promoting effects of oe-circEPHB4 on SOX2 expression. Additionally, in terms of SOX2 mRNA stability, a similar trend was observed in Figure 5M. These evidences suggested that circEPHB4 promotes the high expression of SOX2 as well as maintains SOX2 mRNA stability through its interaction with IGF2BP2.

Overexpressing CircEPHB4 promoted stem properties in glioma cells through SOX2

To investigate whether the effects of CircEPHB4 on glioma stemness depend on SOX2, lentiviruses interfering with SOX2 were constructed. As qRT-PCR experiments revealed

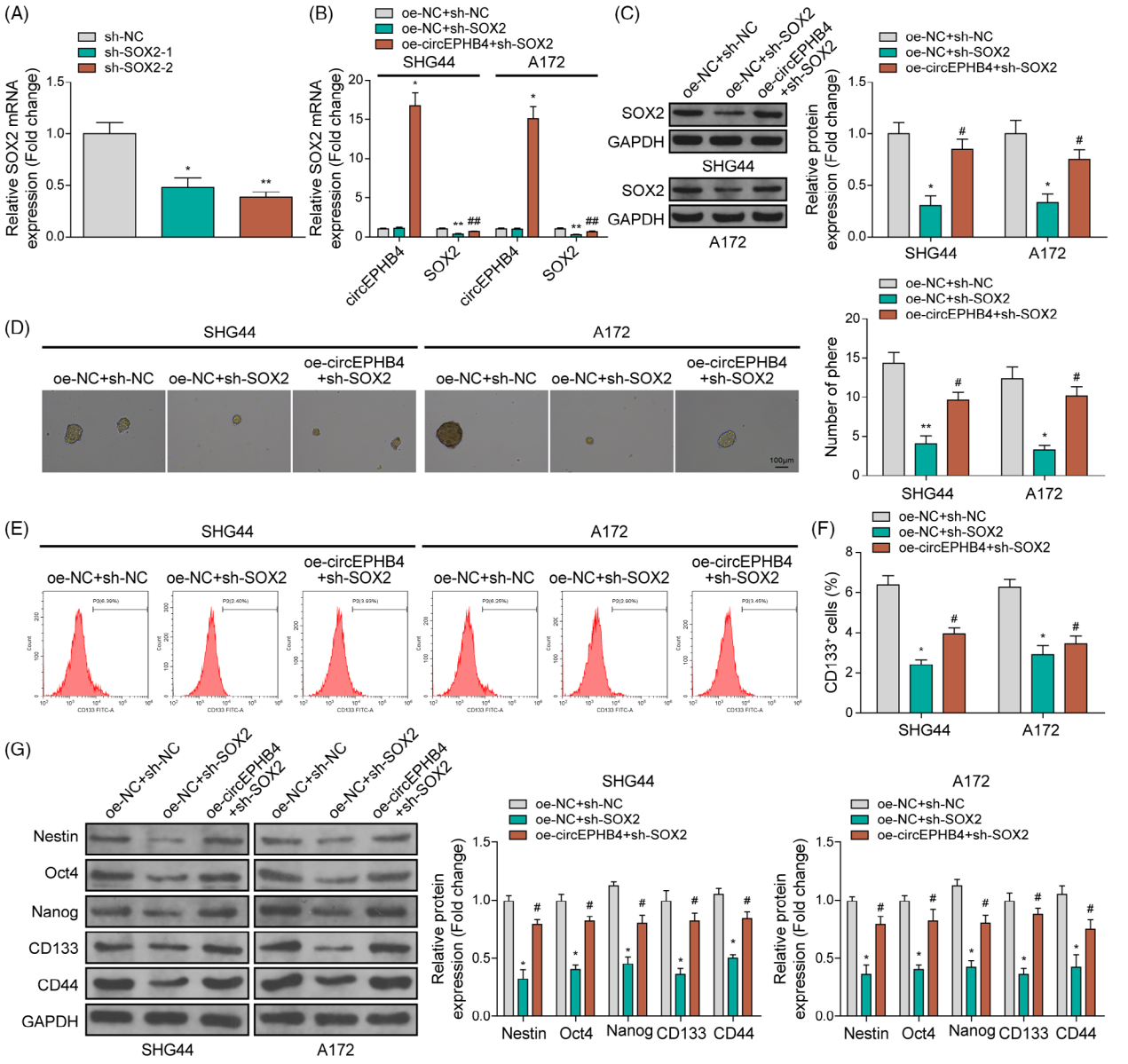

Figure 6. Effect of CircEPHB4/SOX2 axis on stem properties in glioma cells. (A) SOX2 expression in SHG44 via qRT-PCR; *$p < 0.05$ versus sh-NC; (B) CircEPHB4 and SOX2 localization in SHG44 and A172 cells via qRT-PCR; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-SOX2 + oe-NC; (C) CircEPHB4 and SOX2 localization in SHG44 and A172 cells via Western blot; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-SOX2 + oe-NC; (D) Representative images and quantification results of glioma cell spheres formed by SHG44 and A172 cells in cell sphere-forming assay, with a scale of 100 μm; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-SOX2 + oe-NC; (E and F) The positive rate of CD133 on SHG44 and A172 cells detected by flow cytometry; *$p < 0.05$ versus sh-NC + oe-NC, and # indicates $p < 0.05$ compared with sh-SOX2 + oe-NC; (G) Th Related genes' protein levels determined via Western blot; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-SOX2 + oe-NC. The experiments were performed thrice.

better interference of short hairpin (sh)-SOX2-2, subsequent experiments were performed using sh-SOX2-2 (Fig. 6A). Next, SHG44 and A172 cells were infected with lentivirus overexpressing CircEPHB4 and/or lentivirus knocked down for SOX2, and the results indicated suppression of SOX2 mRNA and protein levels by SOX2 knockdown, while further CircEPHB4 overexpression alleviated the inhibition of SOX2 expression by lentivirus infection alone that interfered with SOX2 (Fig. 6B,C). The cell sphere-forming assay exhibited that the knockdown of SOX2 alone reduced the number of glioma cell spheres; however, further overexpression of CircEPHB4 promoted

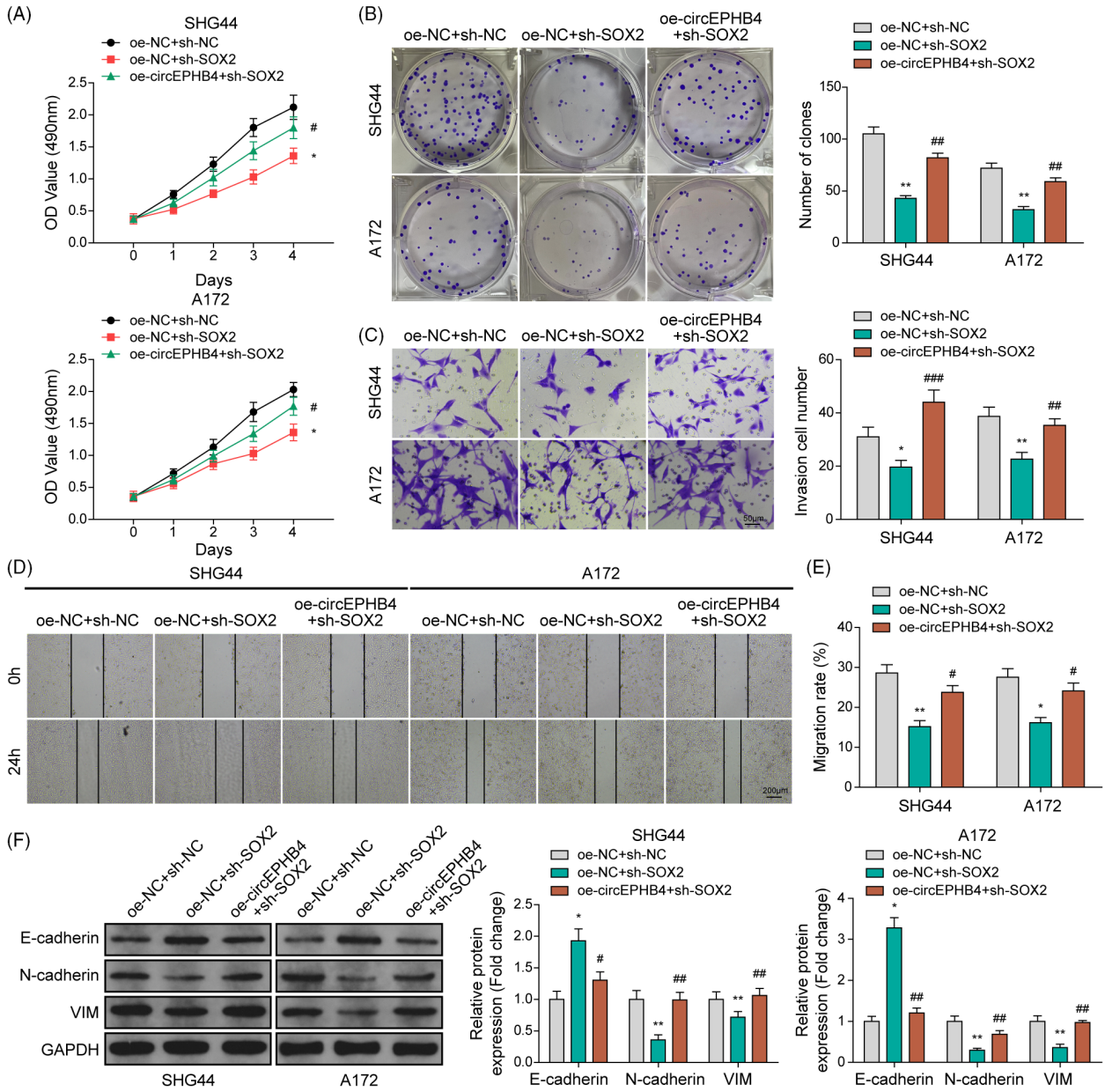

Figure 7. CircEPHB4/SOX2 axis affected the proliferative, invasive and migrative abilities of glioma cells. (A) Cell viability via MTT assay; (B) Cell proliferation via colony formation assay; (C) Cell invasion via transwell assay, with a scale of 50 μm; (D and E) Cell migration via cell scratch assay, with a scale of 200 μm; (F) Related genes' protein levels determined via Western blot. *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-SOX2 + oe-NC. The experiments were performed thrice.

the formation of glioma cell spheres (Fig. 6D). Similarly, CircEPHB4 overexpression remarkably reduced SOX2 knocked-down inhibitory effects on the positive rate of stemness marker CD133 in glioma cells (Fig. 6E,F). Protein analysis further demonstrated that SOX2 knockdown alone reduced Nanog, Nestin, CD133, CD44, and Oct4 levels, while additional CircEPHB4 overexpression depressed those effects(Fig. 6G). Altogether, overexpressing CircEPHB4 promoted glioma stemness through SOX2.

Overexpressing CircEPHB4 eased the inhibitory effects of SOX2 knockdown on glioma cells

Here, we found that CircEPHB4 overexpression lightened the inhibitory effects of SOX2 knocked down alone on glioma cells' viability (Fig. 7A), colony-forming ability (Fig. 7B), and invasion and migration abilities (Fig. 7C–E), as well as decreased E-Cadherin and increased N-

Cadherin and VIM protein levels (Fig. 7F); thereby indicating the promising potential of CircEPHB4 in reversing SOX2 effects to promote glioma cells' proliferation and metastasis.

SOX2 directly bound to the PHLDB2 promoter to promote PHLDB2 transcript expression

PHLDB2 is a key protein with a PH domain linked with the metastatic ability of multiple cells, and its knockdown can promote E-cadherin expression in colorectal cancer cells. JASPAR CORE analysis exhibited a targeted binding site for SOX2 in the PHLDB2 promoter region (Annex 3), while the targeting relationship between the two has not yet been reported. Therefore, we speculated that SOX2 might promote PHLDB2 transcript expression through binding to the PHLDB2 promoter.

qRT-PCR experiments revealed overexpression of PHLDB2 in both glioma cells and tissues (Fig. 8A,B) and that SOX2 mRNA levels positively correlated with PHLDB2 mRNA expression in glioma cancer tissues (Fig. 8C). Further experiments using ChIP assay showed enrichment of SOX2 at the PHLDB2 promoter region of SHG44 cells (Fig. 8D). According to the results of the JASPAR CORE database analysis, we constructed luciferase reporter plasmids of PHLDB2 WT (full-length PHLDB2 promoter sequence) and PHLDB2 mut (PHLDB2 promoter sequence) without binding site to SOX2 (gaacaatgaga), and found that overexpressing SOX2 significantly enhanced PHLDB2 WT group's luciferase signals but not those of the PHLDB2 mut group (Fig. 8E). Furthermore, a significant increase in PHLDB2 mRNA and protein levels in SHG44 cells was detected following the overexpression of SOX2 (Fig. 8F,G). Altogether, we showed an enrichment of SOX2 in the PHLDB2 promoter region, which promoted the transcription of PHLDB2.

Overexpressing SOX2 reversed the inhibitory effects of PHLDB2 knockdown on the self-renewal of glioma cells

Here, we assessed SOX2-regulated PHLDB2 expression effects on glioma cells self-renewal ability. Lentiviruses interfering with PHLDB2 were constructed, and qRT-PCR experiments showed better interference with sh-PHLDB2-1, which was then selected for subsequent experiments (Fig. 9A). Next, SHG44 and A172 cells were infected with lentivirus knocked down for PHLDB2 or lentivirus overexpressing SOX2, and we observed inhibition of PHLDB2 protein expression in glioma cells following PHLDB2 knockdown alone, which could be reversed by overexpressing SOX2 (Fig. 9B). Cell sphere-forming assay demonstrated decreased glioma cell spheres formation following PHLDB2 knocked down,

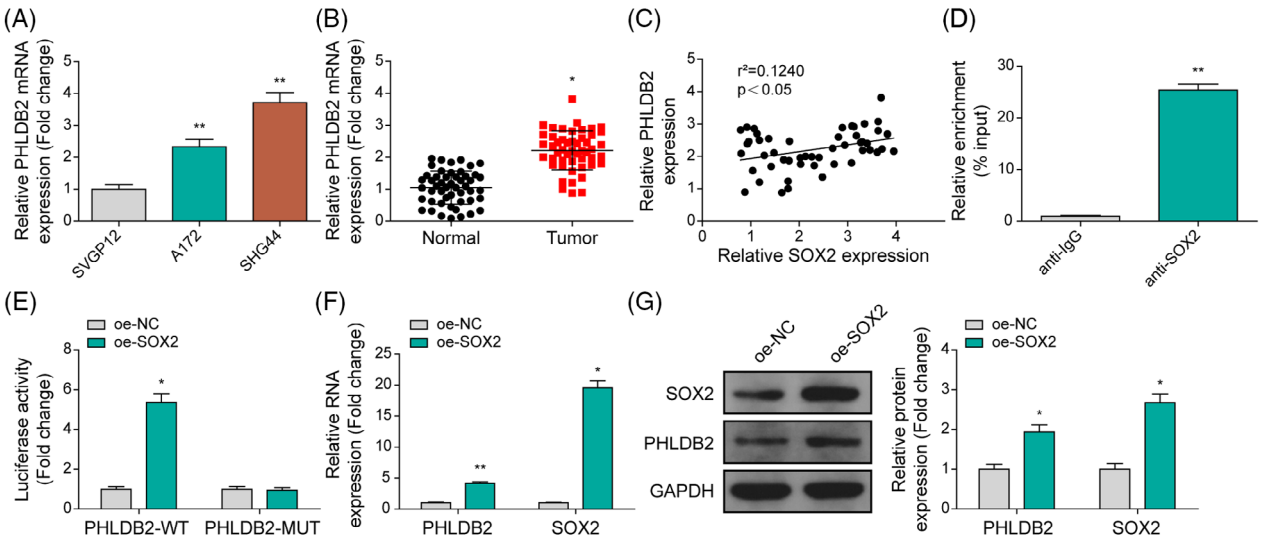

Figure 8. SOX2 binding to the PHLDB2 promoter affected PHLDB2 transcript expression. (A) PHLDB2 expression assessed via qRT-PCR; *$p < 0.05$ versus SVGP12 cells; (B) PHLDB2 expression in glioma and adjacent normal tissues assessed via qRT-PCR; *$p < 0.05$ versus Normal; (C) Association of SOX2 mRNA with PHLDB2 expression in glioma tissues using Pearson correlation coefficient analysis; (D) SOX2 enrichment in the PHLDB2 promoter region of SHG44 cells via ChIP assay; *$p < 0.05$ versus anti-IgG; (E) Targeting relationship between SOX2 and PHLDB2 in SHG44 cells via dual-luciferase reporter assay; *$p < 0.05$ versus oe-NC; (F) mRNA levels of related genes in SHG44 cells via qRT-PCR; *$p < 0.05$ versus oe-NC; (G) Related genes' protein levels in SHG44 cells determined via Western blot. *$p < 0.05$ versus oe-NC. The experiments were performed thrice.

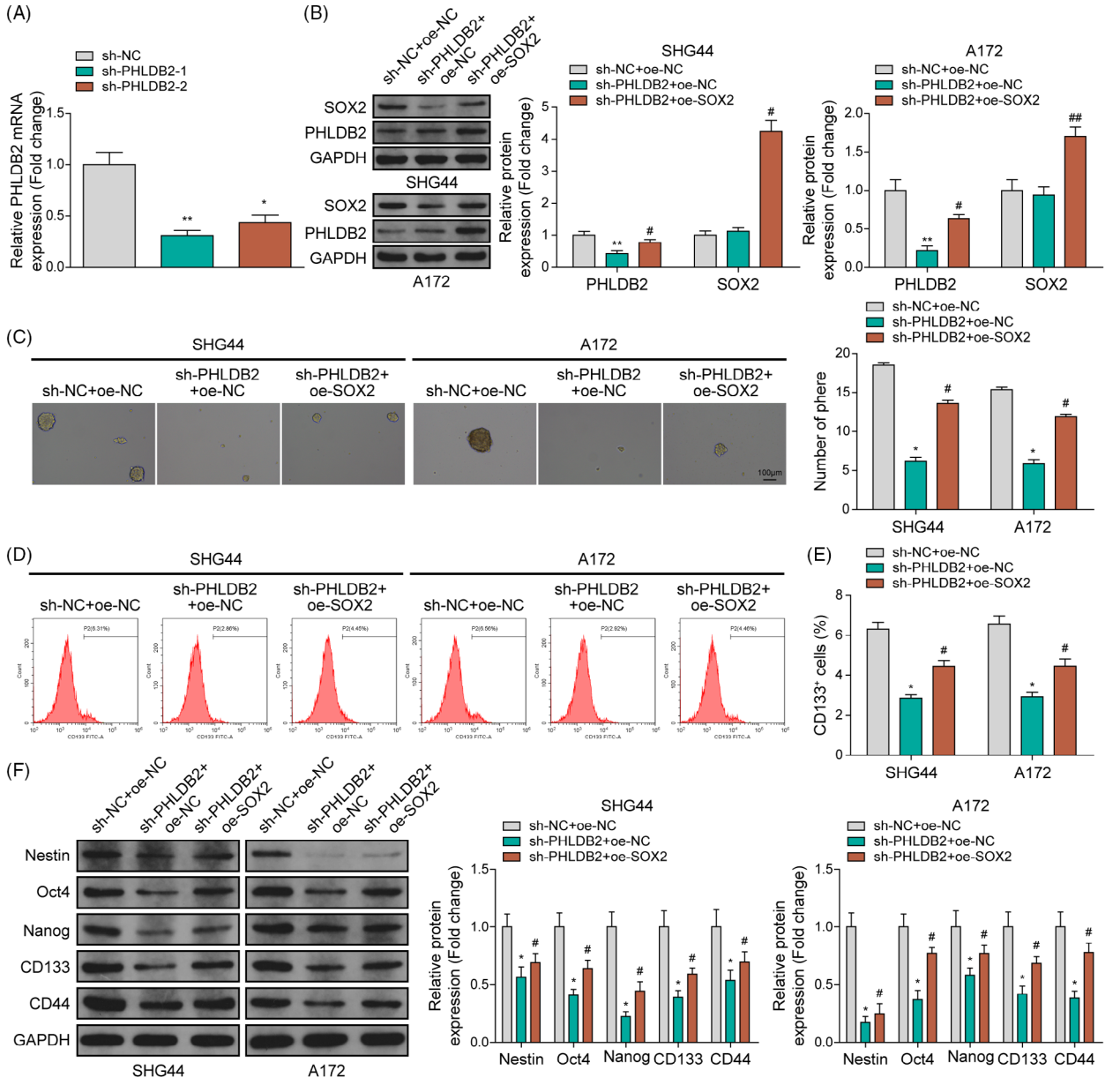

Figure 9. SOX2 affected glioma cell self-renewal by regulating PHLDB2 expression. (A) PHLDB2 expression in SHG44 cells assessed via qRT-PCR; *$p < 0.05$ versus sh-NC; (B) PHLDB2 and SOX2 localization in SHG44 and A172 cells via Western blot; *$p < 0.05$ versus sh-NC + oe-NC, and # indicates $p < 0.05$ compared with sh-PHLDB2 + oe-NC; (C) Representative images and quantification results of glioma cell spheres formed by SHG44 and A172 cells in cell sphere-forming assay, with a scale of 100 μm; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-PHLDB2 + oe-NC; (D and E) CD133 positivity in SHG44 and A172 cells via flow cytometry; *$p < 0.05$ versus sh-NC + oe-NC, and $^{\#}p < 0.05$ versus sh-PHLDB2 + oe-NC; (F) Related genes' protein levels in SHG44 cells determined via Western blot; *$p < 0.05$ versus sh-NC + oe-NC. $^{\#}p < 0.05$ versus sh-PHLDB2 + oe-NC. The experiments were performed thrice.

which could be reversed by overexpressing SOX2 (Fig. 9C). Similarly, flow cytometry showed significant alleviation of PHLDB2 knocked-down inhibitory effects upon overexpressing SOX2 on the positive rate of stemness marker CD133 in glioma cells (Fig. 9D,E). Western blot results indicated that the knockdown of PHLDB2 alone also reduced the levels of Nestin, Oct4, Nanog, CD133, and CD44 in glioma cells, but opposite results could be observed with SOX2 overexpression (Fig. 9F). These experimental outcomes indicated that SOX2 promoted glioma cell self-renewal by promoting PHLDB2 expression.

Overexpressing SOX2 promoted glioma cell proliferation and metastasis by reversing the inhibitory effects of PHLDB2 knockdown

Overexpressing SOX2 reversed the PHLDB2-knockdown-led suppression of glioma cells' viability (Fig. 10A), colony-forming ability (Fig. 10B), and invasion and migration abilities (Fig. 10C–E), as well as increased E-Cadherin and decreased N-Cadherin and VIM levels (Fig. 10F). Thus, SOX2 could enhance the progression of glioma cells *in vitro* by promoting PHLDB2 expression.

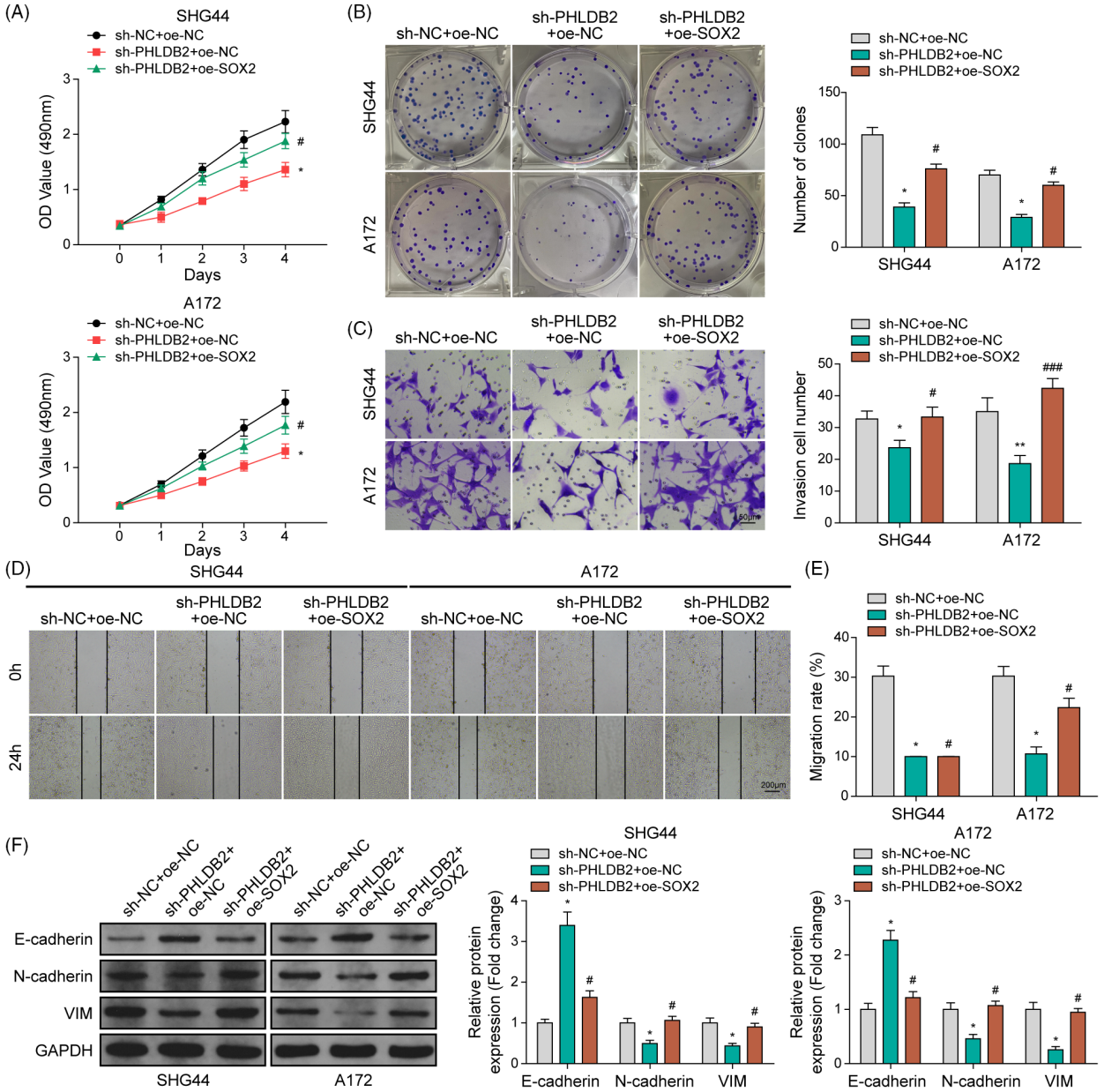

Figure 10. SOX2 affected the proliferation invasion and migration of glioma cells by regulating PHLDB2 expression. (A) Cell viability determined via MTT assay; (B) SHG44 and A172 cell proliferation determined via colony formation assay; (C) The invasion of SHG44 and A172 cells determined via transwell assay, with a scale of 50 μm; (D and E) SHG44 and A172 cell migration detected by cell scratch assay, with a scale of 200 μm; (F) Related genes' protein levels in SHG44 cells determined via Western blot. *$p < 0.05$ versus sh-NC + oe-NC and $^{\#}p < 0.05$ versus sh-PHLDB2 + oe-NC. The experiments were performed thrice.

CircEPHB4 promoted glioma development by regulating the SOX2/PHLDB2 axis

To determine whether the above *in vitro* results could be replicated in vivo, the stably transferred SHG44 cell line was injected into nude mice to construct a glioma xenograft model. Western blot and qRT-PCR results showed that the knockdown of CircEPHB4 alone decreased CircEPHB4, SOX2 and PHLDB2 expressions in the transplanted tumor tissues and increased SOX2 and PHLDB2 expressions in the transplanted tumor tissues after overexpression of SOX2 (Fig. 11A,B). After the knockdown of CircEPHB4 alone, we observed a reduction in tumor volume and weight and increased survival, while SOX2 overexpression significantly inhibited these effects (Fig. 11C–E). Immunohistochemistry assessments indicated decreased protein levels of proliferation marker Ki67 and stemness markers Oct4, Nanog, Nestin, CD44, and CD133 in the transplanted tumor tissues with CircEPHB4 knockdown alone, and opposite results in transplanted tumor tissues with CircEPHB4 knockdown and SOX2 overexpression relative to CircEPHB4 knockdown alone (Fig. 11F). Furthermore, overexpressing SOX2 reduced E-Cadherin and increased N-Cadherin and VIM levels in the transplanted tumor tissues compared to CircEPHB4 knockdown alone (Fig. 11G). Collectively, CircEPHB4 upregulated PHLDB2 expression by stabilizing SOX2 mRNA, thereby promoting in vivo tumor growth and increasing tumor stemness and metastatic abilities.

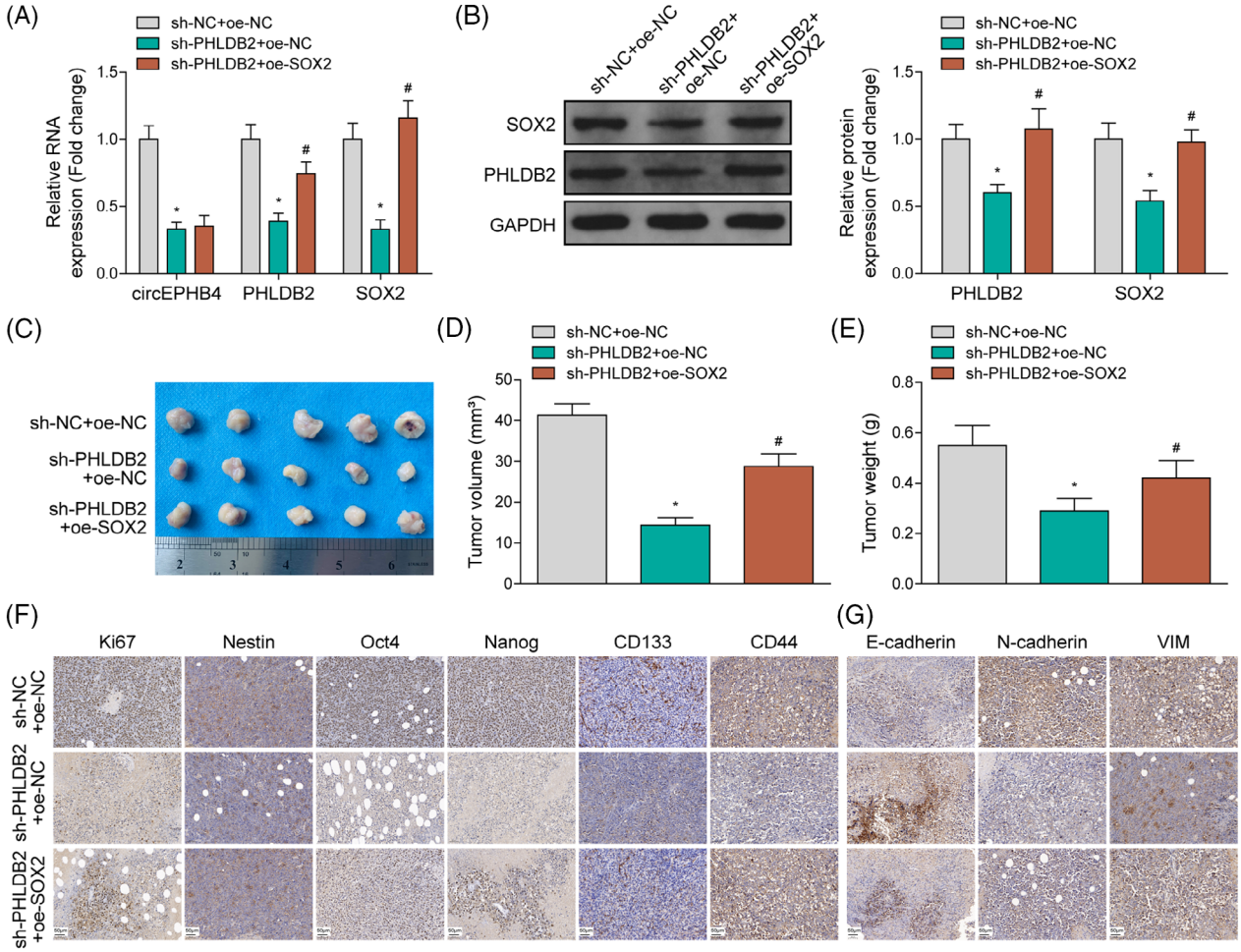

Figure 11. CircEPHB4 affected the development of glioma by regulating the SOX2/PHLDB2 axis. (A) Related genes expressions in brain xenograft tissues assessed via qRT-PCR; (B) The protein expression of related genes in brain xenograft tissues determined via Western blot; (C) Representative image of xenograft tumors; (D and E) Xenograft volume and weight of xenograft tumors; (F and G) Related proteins expressions in xenograft tissues determined via immunohistochemistry, with a scale of 50 μm. $N = 5$, $^{*}p < 0.05$ versus sh-NC + oe-NC and $^{\#}p < 0.05$ versus sh-CircEPHB4 + oe-NC.

Discussion

This study contributes to our understanding of the role of circRNA EPHB4 in glioma pathogenesis by providing evidence of its overexpression in glioma cell lines compared to non-glioma cells. The study also identifies the specific location and cleavage sites of circRNA EPHB4 and demonstrates its stability and presence in both the cytoplasm and nucleus of cells. Furthermore, the findings reveal the involvement of m6A modification and the role of methyltransferase METTL3 and protein YTHDC1 in promoting the cytoplasmic localization of m6A-modified circRNA EPHB4. The study also shows that CircEPHB4 promotes glioma cell self-renewal, cell proliferation, invasion, and migration. Additionally, the interaction between CircEPHB4 and IGF2BP2 is highlighted, indicating that circEPHB4 can increase SOX2 mRNA stability by binding to IGF2BP2, thereby impacting the expression of PHLDB2 through the SOX2/PHLDB2 axis. Overall, these findings provide valuable insights into the molecular mechanisms underlying the involvement of CircRNA EPHB4 in glioma pathogenesis.

CircRNA has been described as an endogenous noncoding RNA.[27] A previous study revealed that circEPHB4 could regulate stem properties and glioma cell proliferation by sponging miR-637 and elevating SOX10 expression.[5] Lu et al. observed that the upregulation of circEPHB4 in glioblastoma promoted the cells' invasion and proliferation abilities via the miR-326/Wnt7B axis.[18] The chemical modification of RNA is an emerging epigenetic regulation in cells and is implicated in various biological processes. m6A modification is considered the most common pattern of post-transcriptional RNA modification in eukaryotes.[28] m6A-modified circEPHB4 refers to the circular RNA molecule circEPHB4 that undergoes m6A modification, where the nucleotide adenosine (A) within the RNA sequence is methylated at the sixth position.[29,30] This modification is catalyzed by specific enzymes and plays a regulatory role in various biological processes, including RNA stability, splicing, localization, and translation.[31] The potential significance of m6A-modified circRNAs lies in their involvement in cancer. Dysregulation of m6A modifications has been implicated in tumorigenesis and cancer progression, as these modifications can influence RNA metabolism and gene expression.[32] Recent evidence indicates that m6A modification is functionally involved in glioma as an oncogenic role.[33] An increasing number of reports show that m6A modification can regulate circRNAs for their enrichment and vital biological functions and their involvement in the pathogenesis of neurological diseases and various cancers.[34] Here, our bioinformatic analysis showed the presence of m6A modification sites on circEPHB4, and was observed to be in both the cytoplasm and the nucleus following *in vitro* experiments and that the YTHDC1 protein promoted the cytoplasmic localization by binding to m6A-modified circEPHB4. Additionally, methyltransferase MELLT3 upregulated the m6A modification of CircEPHB4 and promoted its stability. Moreover, circEPHB4 was highly expressed in glioma cell lines, and further experiments revealed that m6A-modified CircEPHB4 promoted proliferation, metastasis of glioma cells, and self-renewal of glioma cells. These findings, combined with previous evidence, confirm the potential use of circEPHB4 as a biomarker and target in glioma.

Furthermore, bioinformatic analysis using the CircInteractome database identified a binding between CircEPHB4 and the IGF2BP2 protein. Starbase database prediction analysis revealed that IGF2BP2 could bind to SOX2 mRNA. Immunofluorescence and RNA FISH analysis confirmed the co-localization of endogenously expressed CircEPHB4 and IGF2BP2 in glioma cells' cytoplasm. IGF2BP2 was shown to enhance mRNA stability and translation.[35,36] The upregulation of IGF2BP2 has been associated with poor prognosis of low-grade gliomas.[37] SOX2 serves as a transcription factor to maintain essential stem cell properties, including glioma stem cells.[19] Accumulating studies have provided evidence that SOX2 is functionally related to the stemness in glioma cells and unfavorable prognosis in glioma.[20,22] Interestingly, circEPHB4 in the cytoplasm increased SOX2 mRNA stability by binding IGF2BP2. SOX2 overexpression was shown in both glioma cells and tissues, and a positive correlation with CircEPHB4 expression was observed in glioma tissues, which was concordant with the findings of Zhou et al[38] Another study reported that LncRNA PVT1 boosted glioma stemness by mediating the miR-365/ELF4/SOX2 axis.[21] Combined with the above findings and previous evidence, we speculated that SOX2 might enhance the cancerous nature of glioma cells and a potential correlation between SOX2 expression and CircEPHB4. Our experimental results indicated that the knockdown of SOX2 alone inhibited stem of glioma cells and proliferative and metastatic abilities. However, overexpressing circEPHB4 reversed the inhibitory effects of SOX2 knockdown on glioma cells.

Moreover, this study demonstrated that SOX2 and PHLDB2 were positively correlated. PHLDB2 is functionally related to EMT, a critical process for cancer metastasis and stem cells.[23,24] JASPAR CORE analysis revealed a binding site for SOX2 in the promoter region of PHLDB2. Relevant experiments showed overexpression of PHLDB2 in both glioma cells and tissues, and the knockdown of PHLDB2 suppressed stem of glioma cell, proliferation, and metastasis of glioma cells. Similar to our findings, Chen et al. demonstrated an increase in cancer

cell invasion ability upon overexpressing PHLDB2.[39] Furthermore, overexpression of SOX2 reversed the inhibitory effects of PHLDB2 knockdown. Collectively, m6A-modified circEPHB4 promoted SOX2 expression by increasing SOX2 mRNA stability through IGF2BP2 and enhancing SOX2 expression by directly promoting PHLDB2 transcription, thereby strengthening glioma cell stemness and metastasis. Our constructed glioma xenograft model experiment further demonstrated that CircEPHB4 promoted glioma tumor development by regulating the SOX2/PHLDB2 axis.

The clinical and translational impact of these findings can be significant. Firstly, the identification of circRNA EPHB4 as abundantly and stably expressed in glioma cells suggests its potential as a diagnostic biomarker for glioma. Traditional mRNA biomarkers may be susceptible to degradation, limiting their usefulness in diagnostic tests.[40] However, circEPHB4's resistance to mRNA degradation suggests that it could serve as a reliable biomarker for detecting and monitoring glioma, providing clinicians with a stable and robust indicator of disease presence or progression. In addition, its overexpression in glioma cell lines compared to non-glioma cells indicates its potential specificity to glioma and potential use in distinguishing glioma from other brain tumors. Secondly, understanding the regulatory mechanisms of CircRNA EPHB4, such as m6A modification and the involvement of METTL3 and YTHDC1, could offer new therapeutic targets for glioma treatment. The enzymes responsible for m6A modification, known as writers (e.g., MELLT3), erasers, and readers, play crucial roles in regulating m6A-modified circRNAs.[29,41] Developing small molecule inhibitors or specific drugs targeting these enzymes can modulate the m6A modification levels and inhibit the growth and recurrence of glioma by specifically targeting the population of stem cells, which are known to contribute to tumor initiation and therapy resistance. Furthermore, considering that circEPHB4 is resistant to degradation, it may persist in glioma cells and contribute to tumorigenic processes. Thus, targeting circEPHB4 could disrupt its functional roles and potentially inhibit glioma progression or sensitize cells to existing treatment modalities. Third, the findings regarding the role of circEPHB4 in promoting glioma stemness, proliferation, invasion, and migration highlight its importance in glioma pathogenesis and suggest that it could also be useful as a predictive biomarker for treatment response. Changes in the levels of circEPHB4 during treatment may serve as an indicator of treatment efficacy.[5] Decreased circEPHB4 expression following successful treatment could signify positive outcomes, while sustained or increased levels may indicate treatment resistance or disease recurrence. Fourth, the subcellular localization of circEPHB4 may serve as a biomarker for glioma subtypes or disease progression. We hypothesized that differences in the distribution of circEPHB4 between cytoplasmic and nuclear compartments could provide additional molecular characteristics for defining distinct glioma subtypes or predicting patient outcomes. Analyzing the subcellular localization of circEPHB4 in clinical samples may contribute to a more comprehensive understanding of glioma heterogeneity and aid in tailoring treatment strategies based on individualized molecular profiles. Lastly, the insights into the SOX2/PHLDB2 axis provide a deeper understanding of the molecular mechanisms underlying glioma development, and this knowledge may lead to the development of novel therapeutic strategies aimed at modulating this axis for improved patient outcomes. For instance, the stability of circEPHB4 could be leveraged to develop innovative therapeutic delivery systems. CircRNAs have been explored as carriers for delivering therapeutic molecules or siRNAs to target specific genes or signaling pathways.[42,43] Thus, utilizing circEPHB4 as a delivery vehicle could potentially enhance the efficacy and specificity of therapeutic interventions in glioma. However, it should be noted that these are theoretical insights into the potential advancements that could be made in this field, and further in-depth investigations are still required for clarification.

Although our findings provide supportive evidence and show a potential therapeutic target to tackle glioma development and progression, subsequent investigations are still needed to address the study's limitations. First, the study primarily relies on in vitro experiments using glioma cell lines, which may not fully capture the complexity and heterogeneity of gliomas in vivo. Further investigations using larger patient-derived samples and animal models are needed to validate the findings and assess their clinical relevance. Second, while the study highlights the functional roles of CircEPHB4, SOX2, and PHLDB2 in glioma, additional experiments, such as gain-of-function and loss-of-function studies, could further validate their functional significance and strengthen the causal relationship between these molecules and glioma cell behavior. Lastly, exploring other potential targets for circEPHB4 may provide a deeper understanding of the pathogenesis of glioma, and mechanistic studies could also help clarify downstream and upstream targets, which could further help provide more reference for more individualized management of gliomas.

Conclusion

In summary, this study provides insights into the functional interaction and molecular mechanisms of m6A-modified circEPHB4, SOX2, and EphB in glioma. circEPHB4,

regulated by MELLT3 and YTHDC1, promotes glioma stemness, metastasis, and proliferation. It enhances SOX2 mRNA stability through IGF2BP2 interaction, leading to the transcriptional upregulation of PHLDB2 via the PHLDB2 promoter region. Upregulated PHLDB2 contributes to glioma pathogenesis, tumor growth, stemness, and metastasis. Thus, circEPHB4, SOX2, and PHLDB2 could be considered as potential biomarkers and therapeutic targets in glioma.

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Author Contributions

Yuxiang Liao was involved in conceptualization, methodology, visualization, validation, writing—original draft preparation, investigation, and writing—reviewing and editing. Xiaohui Qiu and Zhiping Zhang were involved in data curation. Jingping Liu and Bo Liu were involved in software. Chen Jin was involved in conceptualization, supervision, writing—original draft preparation, and writing—reviewing and editing.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1

Figure S1 m6A methylation levels at CircEPHB4 sites 9 and 19

A-B: m6A methylation levels at CircEPHB4 sites 9 (A) and 19 (B) in each cell line via MeRIP. The experiments were performed thrice.

Figure S2 Transfection efficiency detection of si-METT3, si-YTHDC1, and si-IGF2BP2

SHG44 cells transfected with si-METT3 or si-NC, then mRNA level of METT3 was assessed by qRT-PCR; ** $P < 0.01$ or *** $P < 0.001$ versus si-NC; B: qRT-PCR assay was performed to detect mRNA level of YTHDC1 after SHG44 cells transfected with si-YTHDC1 or si-NC; ** $P < 0.01$ or *** $P < 0.001$ versus si-NC; C: qRT-PCR assay was performed to detect mRNA level of IGF2BP2 after SHG44 cells transfected with si-IGF2BP2 or si-NC; ** $P < 0.01$ or *** $P < 0.001$ versus si-NC. The experiments were performed thrice.

Annex 1 The m6A methylation sites of CircRNA EPHB4 was predicted on the SRAMP website.

Annex 2 CircInteractome database analyses showed that CircEPHB4 could bind to the IGF2BP2 protein.

Annex 3 JASPAR CORE analysis exhibited a targeted binding site for SOX2 in the PHLDB2 promoter region.

Table S1 The sequences of siRNA and shRNA

Table S2 Primer sequences for qRT-PCR

Table S3 Primer sequences for PCR

Table S4 Primer sequences for qRT-PCR (MeRIP)

